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Comprehensive Analysis of Host Cellular Interactions with Human Papillomavirus E6 Proteins Identifies New E6 Binding Partners and Reflects Viral Diversity

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We have begun to define the human papillomavirus (HPV)-associated proteome for a subset of the more than 120 HPV types that have been identified to date. Our approach uses a mass spectrometry-based platform for the systematic identification of interactions between human papillomavirus and host cellular proteins, and here we report a proteomic analysis of the E6 proteins from 16 different HPV types. The viruses included represent high-risk, low-risk, and non-cancer-associated types from genus alpha as well as viruses from four different species in genus beta. The E6 interaction data set consists of 153 cellular proteins, including several previously reported HPV E6 interactors such as p53, E6AP, MAML1, and p300/CBP and proteins containing PDZ domains. We report the genus-specific binding of E6s to either E6AP or MAML1, define the specific HPV E6s that bind to p300, and demonstrate several new features of interactions involving beta HPV E6s. In particular, we report that several beta HPV E6s bind to proteins containing PDZ domains and that at least two beta HPV E6s bind to p53. Finally, we report the newly discovered interaction of proteins of E6 of beta genus, species 2, with the Ccr4-Not complex, the first report of a viral protein binding to this complex. This data set represents a comprehensive survey of E6 binding partners that provides a resource for the HPV field and will allow continued studies on the diverse biology of the human papillomaviruses.

The human papillomaviruses (HPVs) comprise more than 120 different virus types, each with a double-stranded DNA genome of approximately 8 kb. The HPVs have similar genome organizations, with regulatory functions encoded by the early (E) genes and structural components encoded by the late (L) genes (reviewed in reference 24). HPVs of different types differ in DNA sequences by 10% or more in the L1 gene, and the other viral genes exhibit a greater degree of sequence diversity between types (6, 15). Papillomaviruses are grouped into genera based on their L1 gene sequences and are further subdivided into species, with most of the HPVs in genus alpha or genus beta. The genus alpha HPVs infect the mucosal epithelium, and those that are the etiological agent responsible for the development of anogenital cancers (including cervical cancer) fall into genus alpha, species 7, and genus alpha, species 9. Beta-type HPVs infect the cutaneous epithelium.

The HPV E6 protein has long been appreciated as a critical regulator of the viral life cycle and driver of tumorigenesis for the high-risk HPVs. Through the action of the HPV E7 protein, the G_1-S checkpoint is bypassed in infected cells by the inactivation of the retinoblastoma tumor suppressor protein (pRB1) (17, 18, 46). This results in a cellular environment that is conducive to the replication of the viral DNA but in which proapoptotic signals have been triggered. One crucial function of high-risk HPV E6 proteins is to counteract the effects of p53 following this trigger, and this is accomplished by the targeted ubiquitylation of p53 by a protein complex that includes E6 and the ubiquitin ligase E6AP (UBE3A) (54, 69). Although HPV E6s have no intrinsic enzymatic activity, they provide many other functions in the HPV-infected cell, and those that have been described to date are largely the result of protein-protein interactions (PPIs) involving E6. Some of these PPIs are restricted to E6s from one or two PV species. For example, proteins containing PDZ domains have been shown to bind only to HPV E6s from the high-risk viruses in genus alpha, species 7 and 9. The unstructured C terminus present in these E6s contains a PDZ binding motif (PBM) with the amino acid sequence X-T-X-L/V_{COOH} (33, 38).

Other interactions, such as the interaction of E6AP with E6 proteins from both high- and low-risk species of genus alpha, are more broadly conserved (9, 26). It is curious that the relatively well-conserved E6-E6AP interaction has different consequences for the high- and low-risk HPVs, since it results in the degradation of p53 only in the case of the high-risk E6 proteins.

Other known interactions follow different patterns. E6s from HPV type 16 (HPV16), -18, -11, -5, -8, and -38 as well as those from cottontail rabbit papillomavirus (CRPV) and bovine papillomavirus type 1 (BPV1) have been reported to bind to the acetyltransferases CBP and/or p300, although this has this been demonstrated convincingly only for HPV16, -5, and -8 E6s by a coimmunoprecipitation of endogenous p300 from E6-expressing cells (23, 44, 49, 61, 71, 72). The downstream effects of the 16E6-p300/CBP interaction have been proposed to include inhibition of p53 and NF-κB transcription and an ultimate inhibition of cellular differentiation (49) and a downregulation of p53 activity and p53-dependent transcription (61, 71). These functions are in contrast to the proposed effects of p300 binding to HPV5 and -8 E6s, which are the degradation of p300 and the altered expression of

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cellular differentiation markers resulting from E6 blocking the association of Akt with p300 (23).

Recent work from several laboratories has begun to focus on the diverse activities, characteristics, and PPIs of the HPV E6 proteins. Mesplède and colleagues examined the ability of 29 different E6s, most from genus alpha, to target p53 for degradation and characterized their subcellular localization patterns (43). A separate study by Cornet and coworkers characterized the ability of various beta HPV E6 proteins to increase the life span of human keratinocytes and to alter p53 functions in beta HPV E6-expressing cells (14). Additionally, Rozenblatt-Rosen and colleagues recently reported both yeast two-hybrid and affinity purification-mass spectrometry (MS) analyses that examined the binding partners of proteins, including E6 and E7, from four alpha genus and two beta genus HPVs (52).

While the causative role of alpha HPVs and alpha HPV E6s in cancer has been firmly established, the comparative studies and other reports in the literature have raised, but not answered, questions about the capacity of the beta HPVs and their oncoproteins to immortalize and transform cells. Several of the beta HPVs are those that are found in hyperproliferative cutaneous lesions characteristic of the genetic disease epidermodysplasia verruciformis (EV). Once an EV patient has reached middle age, the likelihood that some of these lesions, especially those located in sun-exposed regions, will have progressed to squamous cell carcinoma (SCC) is quite high (50). An additional postulated link between beta HPVs and cancer comes from the observation that HPV DNA is frequently detected in nonmelanoma skin cancers (NMSC) (27, 50), and it is found even more often in the actinic keratoses that can precede and progress to SCC (2). Healthy skin samples, however, also frequently contain beta HPV DNA, and HPV transcripts are detected at a remarkably low level in SCCs (2, 4, 19). If beta HPVs have a role in carcinogenesis, it may be more related to initiation rather than to maintenance of the transformed state.

To characterize and better understand the diversity among the HPVs and to determine what features of different HPV-encoded proteins determine the differences in their biology, we have established a platform for the systematic identification of interactions between HPV and host cellular proteins (70). Viral proteins from a representative subset of the HPVs are epitope tagged, stably expressed in human keratinocytes, and analyzed by immunoprecipitation, mass spectrometry, and the Comparative Proteomic Analysis Software Suite (CompPASS). Using this unbiased detection of protein-protein interactions (PPIs), we were able to identify and characterize both conserved and type-specific interactions of cellular proteins with HPV E7 proteins from 17 different virus types. This suggests that the sequence differences that result in the HPV phylogeny are also reflected by differential PPIs that have the potential to explain aspects of HPV biology.

A similar approach for other viruses has proved equally useful. A recent report defined the HIV proteome and noted that only 4% of the interactions defined in that mass spectrometry-based study had been previously reported in the VirusMINT database, highlighting the need for such global analyses (28). Other groups have reported the mapping of PPIs for HPV16 (21) or for E2 from several HPV types (45) by yeast two-hybrid analyses. The yeast two-hybrid approach may reveal new interactions but lacks the potential to address which proteins are expressed in the natural host cell type of HPV infection and cannot detect multiprotein complexes.

The conserved binding of pRB1 by E7s discussed above suggested that aspects of the mechanism by which HPVs bypass the G_1-S checkpoint in order to promote viral DNA replication are universal. We speculated, though, that the mechanisms by which HPVs respond to the signals triggered by pRB1 inactivation might differ by virus genus, species, or type. We therefore continued our analysis of HPV-host PPIs by conducting an analysis of the binding partners of the HPV E6 protein. This report describes our E6 proteomic analysis and the interactions we identified. We present a data set that was generated using 16 different HPV E6 proteins in which we detected 153 cellular proteins in complex with at least one E6. These cellular proteins include previously known E6 interactors as well as novel binding partners, and subsets of the interactions are restricted to a defined HPV genus, species, or type.

MATERIALS AND METHODS

Cloning and plasmids. Sixteen different HPV E6 open reading frames (ORFs) were subcloned from genomic plasmids into pDONR223 and then recombined into pMSCV-N-HA-IRES-PURO as previously described (70). To generate mutants of HPV16 E6, pDONR223-16E6 was used as the template for site-directed mutagenesis performed by standard techniques. In the 16E6 8S9A10T mutant, the amino acids R-P-R at positions 8 to 10 are replaced by S-A-T (32). The 16E6 I128T mutation was generated as described in reference 40. The 16E6 Δ146–151 mutant was generated by deleting the nucleotides encoding the last 5 amino acids (aa) of the full-length protein (33). The HPV18 E6 nonsplice (ns) changes were made to the reported splice donor sites in that protein (12) but using the sequence change shown (Fig. 1C). This introduced a change consistent with a published 16E6 ns mutant (55), which as a consequence of disrupting the splice site also introduces the amino acid change V42L (V44L in HPV18 and HPV45 E6). It is not possible to disrupt the splice site without introducing an amino acid change, but each of the 16 different E6s in the study has a V, I, or L at this position (Fig. 1A). The HPV45 E6 ns mutant was generated based on the HPV18 E6 sequence. HPV16 E6 mutants as well as the wild-type control were recombined into pMSCV-N-FLAG-HA-IRES-PURO for mammalian expression. The HPV16 E7-FlagHA expression vector and cell line have been previously described (70). For expression of untagged E7 in the presence of HA-E6, three E7 ORFs were subcloned with a STOP codon into pDONR223 and then recombined into pMSCV-C-FlagHA-Blasticidin as described above. Thus, the C-terminal epitope tags encoded by this vector are not expressed, and cell lines that coexpress HA-E6 and untagged E7 and that are resistant to both puromycin and blasticidin were generated. Plasmid sources and details are summarized in Tables S1 and S2 in the supplemental material.

Tissue culture and cell lines. N/Tert-1 cells were infected with E6 retroviruses, selected, propagated in keratinocyte serum-free medium (K-SFM), and grown to high density in a mix of K-SFM and DF-K medium as described previously (16, 70). For MG132 treatment, cells were treated with 30 μM MG132 or a dimethyl sulfoxide (DMSO) control in culture medium for 4 h prior to harvest. For cycloheximide treatment, cells were treated with 40 μg/ml cycloheximide in culture medium and harvested at the indicated time points.

Immunoprecipitation. Hemagglutinin (HA)-tagged proteins were immunoprecipitated as previously described (5, 57, 70). Briefly, the cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40), with freshly added protease and phosphatase inhibitors (Roche Complete, EDTA-free protease inhibitor cocktail, 25 mM sodium fluoride, 1 mM sodium orthovanadate, and 5 mM ß-glycerophosphate). HA-tagged proteins were purified with anti-HA resin (Sigma A2095). For mass spectrometry analysis, protein-HA resin complexes were washed three times in phosphate-buffered saline (PBS), eluted with HA peptide, and trichloroacetic acid (TCA) precipitated as described previously (5, 57). For Western blot analysis, proteins were eluted from the HA beads by boiling in 1× reducing sample buffer (50 mM Tris [pH 6.8], 0.2% sodium

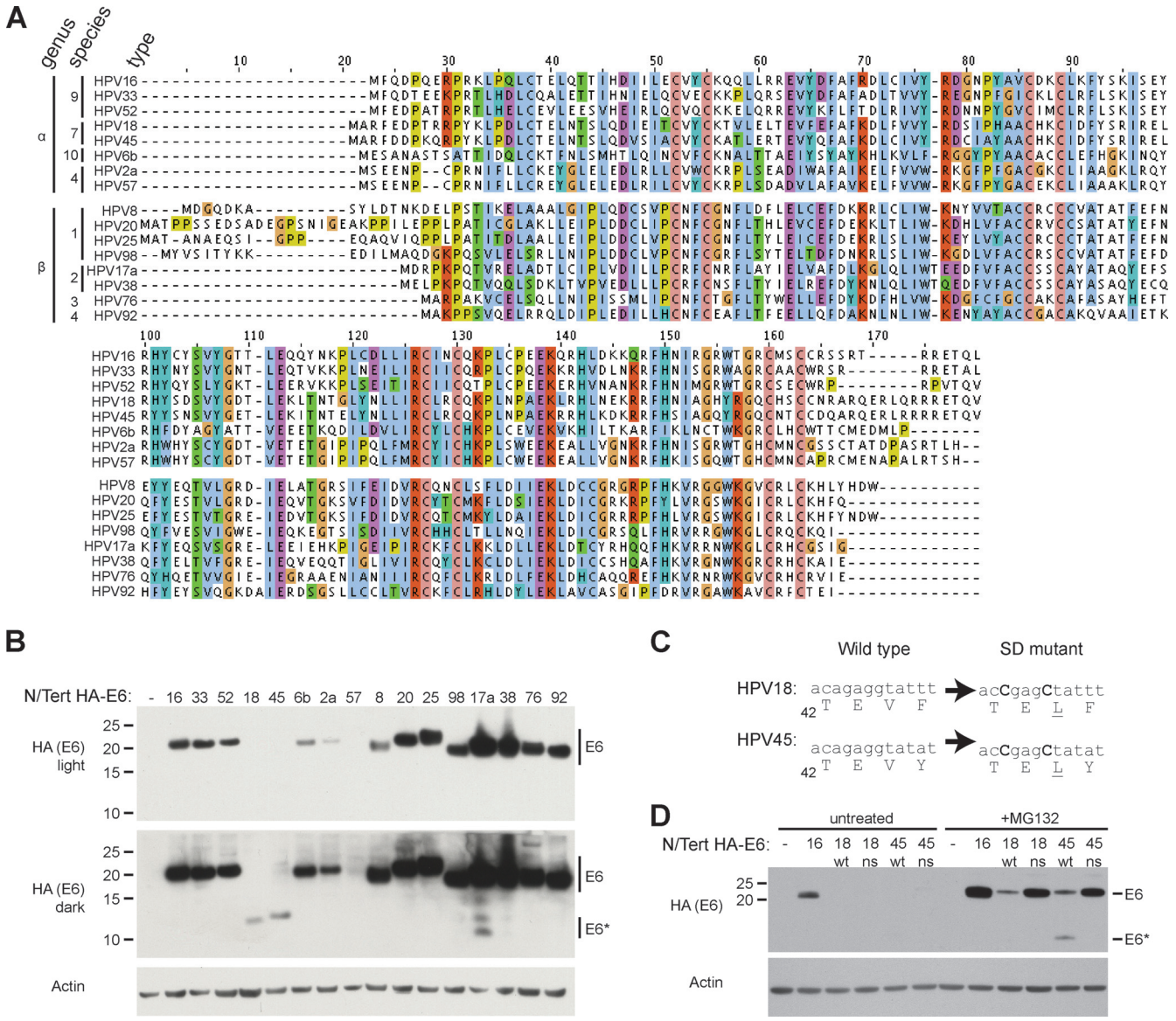

FIG 1 N/Tert-1 cell lines express HA-HPV E6 proteins from 16 virus types. (A) Amino acid sequence alignment of 16 E6 ORFs used in this study. Sequences were aligned by ClustalW and amino acids colored according to the ClustalX color scheme. (B) Western blot of stable E6 expression in N/Tert-HA-E6 stable cell lines, detected with anti-HA antibody. (C) Sequence changes made in HPV18 and 45 E6 ORFs to disrupt the predominant splice donor (SD) site and produce full-length E6. (D) Expression of HPV18 and -45 E6 wild-type (wt) or nonsplice (ns; splice donor site mutated) proteins. N/Tert-1 stable cells were treated with 30 μM MG132 or with DMSO as a control for 4 h and then harvested and analyzed by Western blotting using anti-HA and -actin antibodies.

dodecyl sulfate, 10% glycerol, 5% 2-mercaptoethanol, 25 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM ß-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 50 μM leupeptin, 100 μM pepstatin A).

Mass spectrometry and data analysis. Trypsin digestion, stage tip purification, and mass spectrometry analysis of immunoprecipitated proteins were performed as described previously (5, 57). CompPASS analysis was performed as previously published using a statistics (stats) table containing interaction data generated from HA immunoprecipitations of 39 stable cell lines, each expressing a single HA-tagged human protein bait. Cell lines used in the stats table are the same as those previously reported (70), with the following changes: N/Tert-1-UBE3A and -UCHL5 cell lines were not included, and N/Tert-1-RB1 and SHSY5Y-ECH1, -NCOA4, and -MAPK6 cell lines were included to increase the diversity of the stats table. In these analyses, a protein identified by MS/MS is considered to be a high-confidence cellular interacting protein (HCIP) if its weighted D-score (WD-score—a metric that considers the uniqueness and the abundance of the interactor and the reproducibility of the interaction) is greater than 98% of the WD-scores in the stats table, unless otherwise noted. A normalized WD-score (NWD-score) of ≥1 denotes an HCIP. For interactome analysis, the interactions among the HCIPs from the HPV E6 immunoprecipitation-MS/MS analyses were determined using the STRING database with a score cutoff of 700. The resulting interactions were visualized using Cytoscape (56).

Western blotting. Proteins were separated on NuPAGE (Invitrogen) or SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF). After blocking in 5% nonfat dried milk–TBS-T (Tris-buffered saline [pH 7.4] with 0.05% Tween 20) was performed, blots were incubated with primary antibodies as follows: actin (Millipore), HIF1α (Cell Signaling Technology), p300 (BD Biosciences), E6AP (Sigma), MAML1 (Cell Signaling Technology), CNOT1 (gift of Martine Collart), CNOT2 (Bethyl),

CNOT3 (Bethyl), PTPN13/FAP1 (Santa Cruz Biotechnology), Paxillin (Upstate), p53 (Santa Cruz Biotechnology), MDM2 (Millipore), or Scribble (Cell Signaling Technology). Membranes were washed in TBS-T and incubated in horseradish peroxidase (HRP)-coupled anti-mouse or anti-rabbit antibodies or an Alexa-680-coupled anti-mouse antibody and detected using a Western Lightning chemiluminescent substrate or a Li-COR Infrared imaging system. HA-tagged proteins were detected using an HA antibody conjugated to HRP (Roche) and visualized on film.

RESULTS

Unbiased identification of HPV E6 interactions with host cellular proteins. We previously established a system for the unbiased identification of HPV-host cellular protein-protein interactions (PPIs) in the natural host cell type of the HPV infection. This system uses HA-tagged HPV bait proteins expressed in Tert-immortalized N/Tert-1 human keratinocytes (16). These cells are then processed for HA immunoprecipitation followed by mass spectrometry and analysis with CompPASS software, such that a list of high-confidence cellular interacting proteins (HCIPs) is generated for each HPV bait.

We began by cloning 17 different HPV E6 open reading frames (ORFs) as previously described (70). These included nine E6s from genus alpha, including HPV16, -31, -33, and -52 (high-risk, species 9), HPV18 and -45 (high-risk, species 7), HPV6b (low-risk, species 10), and HPV2a and -57 (species 4), and eight E6s from genus beta, including HPV8, -20, -25, and -98 (species 1), HPV17a and -38 (species 2), HPV76 (species 3), and HPV92 (species 4). We generated N/Tert-1 stable cell lines expressing each E6 protein. In preliminary studies, we were not able to detect the expression of HA-tagged HPV31 E6, an observation that is consistent with the published report of Lee and Laimins (37). We therefore continued the study with the 16 remaining E6s (Fig. 1A). Several sequence elements are conserved among all of the E6s, including the four CXXC zinc binding motifs, while other sequences diverge.

Each of the HA-tagged E6s could be detected in the N/Tert-1 cell lines by the use of an anti-HA immunoblot, although the expression levels of the various E6s differed (Fig. 1B). Since each E6 is expressed from the same promoter, we believe that these differences in expression level stem largely from inherent differences in E6 protein stability (see Fig. 8). We noted that the E6 cell lines of genus alpha, species 7 (HPV18 and HPV45 E6), expressed only a smaller form of E6 that we speculated was the spliced E6* protein. To address this issue, we made conservative mutations to the splice donor sites in each of these ORFs (Fig. 1C) that are consistent with mutations reported in the literature (12, 55) and described further in Materials and Methods. The resulting modified ORFs express 18E6 and 45E6 proteins that are full length, albeit perhaps still relatively unstable, as their expression was more readily detected by Western blot analysis following inhibition of the proteasome via treatment with MG132 (Fig. 1D). These modified E6s are referred to as HPV18 or HPV45 E6 nonsplice (ns) and were used throughout the remainder of the study.

We next processed the 16 different E6-expressing cell lines for protein interaction analysis. HA-tagged E6 was immunoprecipitated from each N/Tert-1 cell line and processed, and data were analyzed as described in Materials and Methods and references 5, 57, and 70. Each cell line was analyzed at least twice, each time in both the presence and the absence of the proteasome inhibitor MG132. Proteasome inhibition was included in order to better detect binding events in which the cellular interactor is targeted for degradation by its association with E6 (e.g., p53 or proteins that contain PDZ domains). This generated a list of 153 unique cellular proteins with which one or more of the E6s interact (see Tables S3 to S5 in the supplemental material). An initial, global analysis of the data (Fig. 2; see also Fig. S1 in the supplemental material) suggested that within the data set there exist cellular proteins that interact with all E6s of a given genus, interactors that are restricted to a single species, or interactors that interact only with HPV E6 from a single virus type.

Analysis of known interacting proteins binding to diverse E6 baits reveals novel interaction specificities. The E6 interactor data set was next examined for the presence of known E6-interacting proteins. While these proteins were already known to interact with one or more E6s from different virus types, here we present a comprehensive report of binding to the 16 different E6s, in several cases revealing new binding patterns. Both our laboratory and that of Scott Vande Pol have previously reported that HPV E6s from genus beta virus types bind to the transcriptional coactivator MAML1 via the LXXLL motif present in MAML1 (10, 60). HPV16 E6 does not bind MAML1, and the downstream effect of the beta E6 interaction is an inhibition of MAML1-mediated Notch signaling. We also note that Rozenblatt-Rosen and colleagues have recently reported results of a study examining the binding of MAML1 to E6 proteins from six different HPV types. In their study, MAML1 bound to the two genus beta E6s but not to any of the four genus alpha E6s that were tested (52).

Consistent with these earlier reports, we found that neither MAML1 nor its binding partners Notch1, Notch2, and RBPJ were detected in complex with any of the eight alpha genus E6s in the study but that MAML1 did bind to the majority of the beta E6s tested (Fig. 2A, top panel). In contrast, E6AP (UBE3A) bound to each of the genus alpha E6s but not to any of the genus beta E6s, although we note that the NWD-scores for the interaction with species 4 E6s (from HPV2a and -57) are below the NWD = 1 significance threshold in this analysis. We validated the genus alpha-specific interaction of E6 with E6AP and the genus beta-specific interaction of E6 with MAML1 by immunoprecipitation of HA-tagged E6s from N/Tert-1 stable cell lines and Western blotting for the endogenous cellular protein (Fig. 3).

We continued by examining the pattern of binding for other proteins known to interact with E6AP or to be targeted for degradation by the high-risk E6-E6AP complex. HERC2 is known to bind to E6AP (35, 42) and was detected as an HCIP with NWD-scores ≥ 1 only for E6s from genus alpha. In most cases, the interaction of E6 with HERC2 had a higher NWD-score when cells were treated with MG132 than when they were treated with the DMSO control. p53 was detected in complex with several high-risk alpha E6s, again with higher NWD-scores in the presence of MG132. In contrast to HERC2, p53 also bound to two E6s from genus beta HPVs: HPV38 and HPV92. To our knowledge, this is the first report of E6s from genus beta virus types interacting with p53, and this result is examined in greater detail below.

Next, we examined the data set for interactions between E6s and proteins that contain a PDZ domain. As expected, several high-risk genus alpha E6s bound to PDZ proteins, including PTPN3, Scribble, and GOPC (CAL) (Fig. 2A, top panel). Each of these interactions had been previously reported for at least one high-risk HPV E6 (30, 31, 47, 65). Surprisingly, several genus beta E6s also bound to proteins containing PDZ domains, including PTPN13 and PDZD11, in some cases with NWD-scores greater

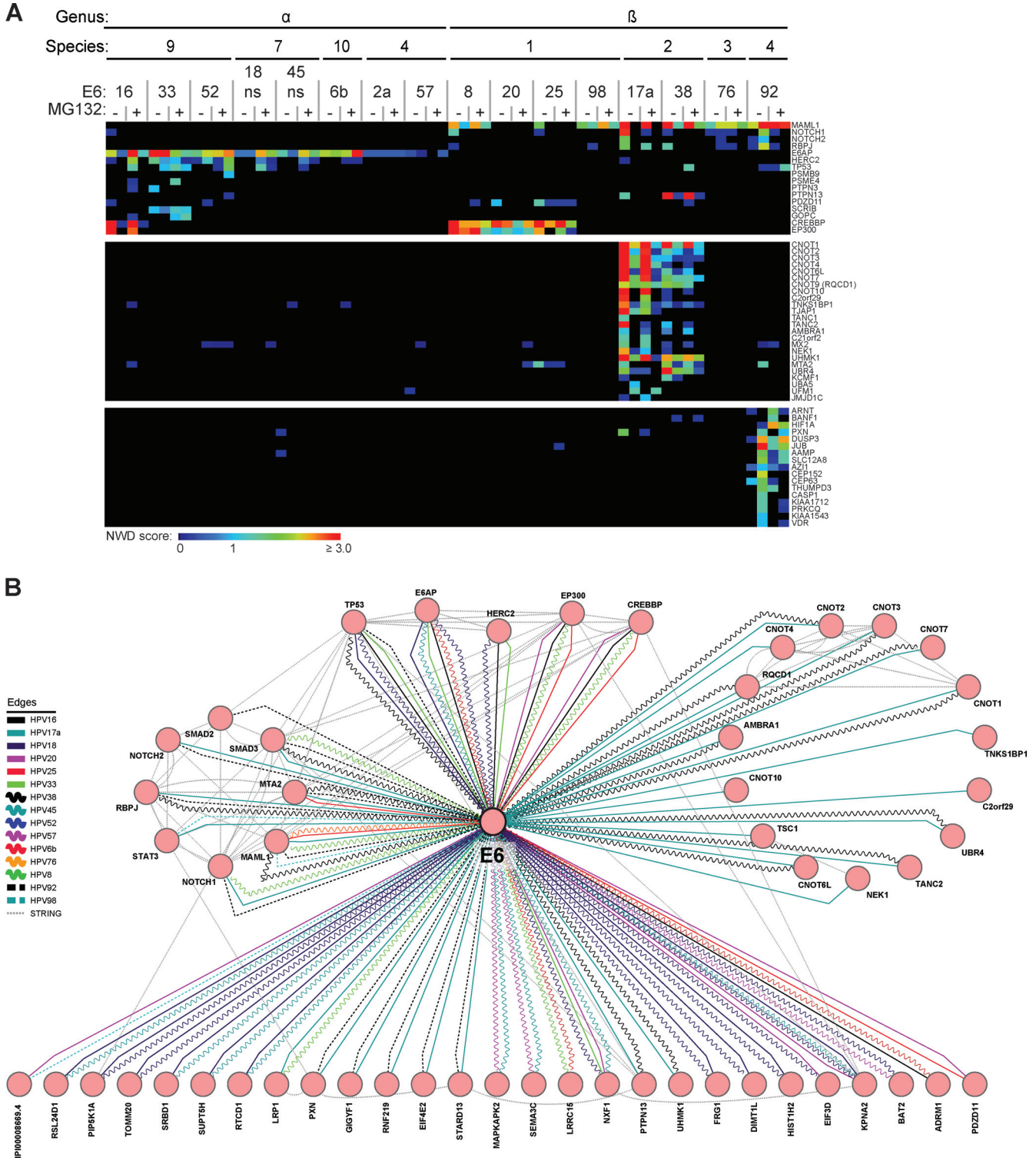

FIG 2 Selected HCIPs of HPV E6s. (A) Heat map representing protein-protein interactions identified by immunoprecipitation-MS/MS and CompPASS analysis when one of 16 unique HPV E6 proteins was used as a bait. Colors in the heat map represent NWD-scores, where an NWD-score ≥ 1 defines a high-confidence interaction. Each column in the heat map represents a replicate experiment. Cells were treated for 4 h with 30 μM MG132 (+) or DMSO control (−) prior to harvest. E6 baits were ordered across the top of the map according to the established HPV phylogeny (6); HCIPs were arranged using a Manhattan distance hierarchical clustering analysis. Selected interactions discussed in the text are shown. (B) Interactome representing HCIPs in complex with various HPV E6 proteins. An interaction is displayed in the heat map when it was detected with two or more HPV E6 bait proteins and had an NWD-score ≥ 1. Dashed lines represent interactions reported in the STRING database.

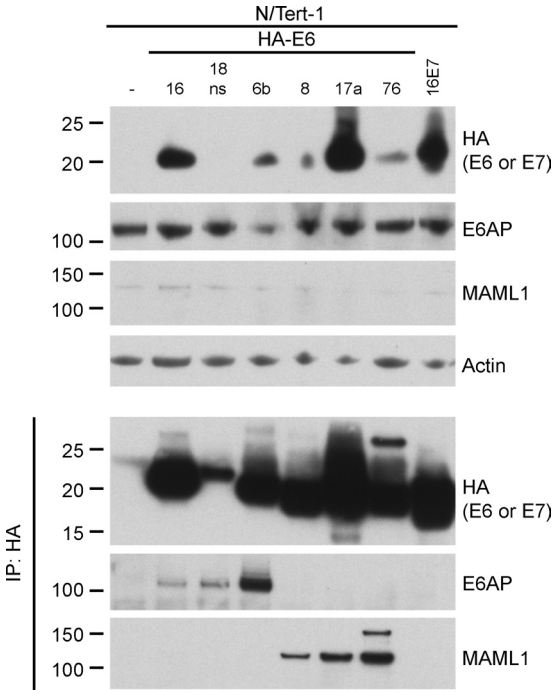

FIG 3 E6AP and MAML1 bind to HPV E6 proteins in a genus-specific manner. N/Tert-1 cells expressing HA-E6 or HPV16 E7-Flag HA (as a control) were subjected to immunoprecipitation with HA antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting using antibodies to HA, actin, E6AP, and MAML1. Top panels, whole-cell lysates. Bottom panels, anti-HA immunoprecipitate (IP: HA).

than those for the alpha E6-PDZ interactions. In general, the NWD-scores for the beta E6-PDZ protein interactions did not increase with MG132 treatment. We further confirmed one of these interactions, HPV38 E6 binding to PTPN13/FAP1, by immunoprecipitation-Western blot analysis (Fig. 4A). Although PTPN13 has been previously shown to bind to HPV16 E6 and target it for degradation (58), we did not observe the HPV16 E6-PTPN13 interaction even after MG132 treatment. We hypothesize that differences in the glutathione *S*-transferase (GST) pulldown approach used in that report and the coimmunoprecipitation from keratinocytes used here may explain the differences in binding patterns. PDZD11 has not previously been reported to bind to any E6.

Finally, various publications have reported the interaction of E6 with the acetyltransferases p300 and CBP (23, 44, 49, 71, 72). Our comprehensive analysis showed that among the genus alpha E6 proteins, HPV16 E6 is unique in its ability to bind to both CBP and p300 (Fig. 2A, top panel). Even other closely related species 9 E6s did not bind to either acetyltransferase. We note that the species 7 E6 proteins, from HPV18 and HPV45, are expressed at a lower level than the other genus alpha E6 proteins. As a result, we cannot rule out the possibility that, with increased expression of these baits, we would detect binding of p300 to HPV18 and/or HPV45 E6. For genus beta E6 proteins, binding to CBP/p300 was nearly conserved across species 1, as an interaction that does not appear to be significantly altered by the addition of MG132 was detected for HPV8, -20, and -25 E6s but not for HPV98 E6. We confirmed the restricted binding of p300 to HPV16, -8, and -25 E6s by immunoprecipitation-Western blot analysis (Fig. 4B).

During an HPV infection, E6 and E7 proteins are expressed at the same time. It is possible that the presence of E7 in cells might alter the profile of cellular proteins to which E6 binds and that coexpression of E7 would allow us to generate E6 interaction data that better reflect the conditions in infected cells. To test this hypothesis, we infected a subset of the E6 cell lines described above with retroviruses expressing untagged E7 proteins from the same HPV type. We thus generated three additional cell lines, N/Tert-1 HA-16E6 plus Untagged-16E7, N/Tert-1 HA-33E6 plus Untagged-33E7, and N/Tert HA-2aE6 plus Untagged-2aE7. We processed these for immunoprecipitation-MS/MS and CompPASS as

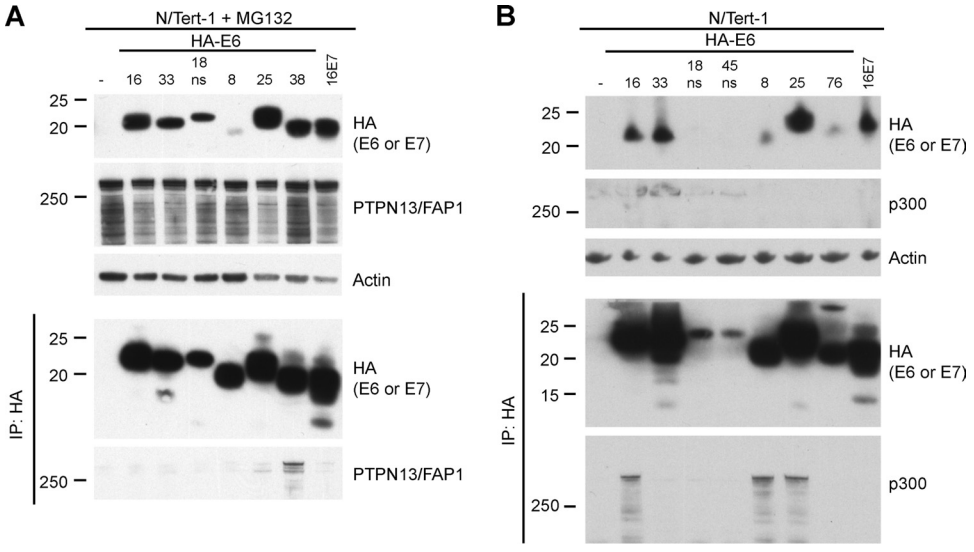

FIG 4 HPV type-specific interactions with PTPN13 and p300. N/Tert-1 cells expressing HA-E6 or HPV16 E7-Flag HA (as a control) were subjected to immunoprecipitation with HA antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting using antibodies to HA, actin, PTPN13 (A), and p300 (B). Top panels, whole-cell lysates. Bottom panels, anti-HA immunoprecipitate. Cells in panel A were treated with 30 μM MG132 for 4 h prior to harvest. Top panels, whole-cell lysates. Bottom panels, anti-HA immunoprecipitate.

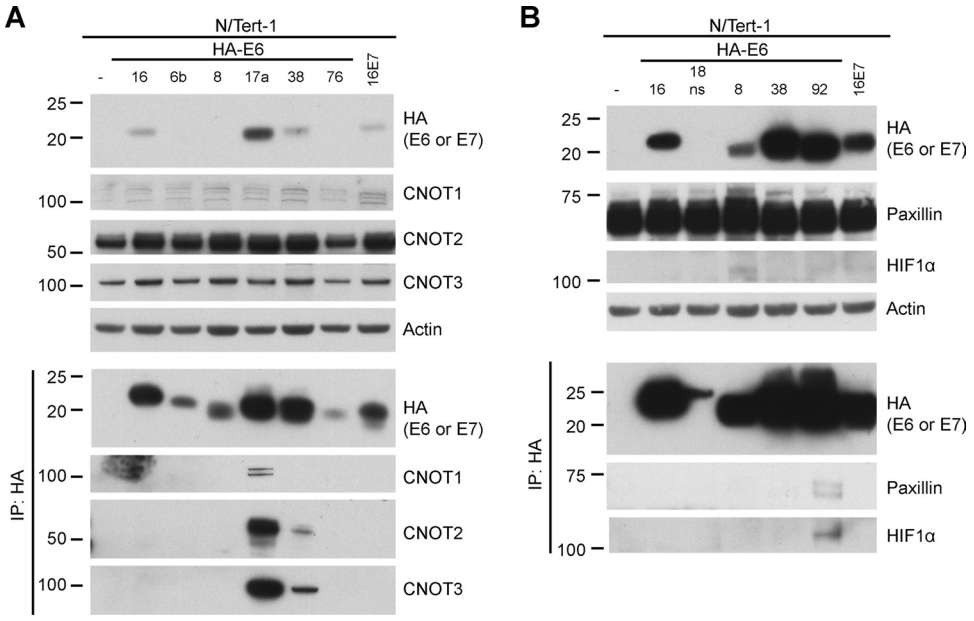

FIG 5 Novel species-specific interactions with genus beta HPV E6 proteins. N/Tert-1 cells expressing HA-E6 or HPV16 E7-Flag HA (as a control) were subjected to immunoprecipitation with HA antibody. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blotting using antibodies to HA, actin, CNOT1, -2, and -3 (A) or Paxillin and HIF1α (B). Top panels, whole-cell lysates. Bottom panels, anti-HA immunoprecipitate.

described above and compared the resulting data to the data generated for the corresponding E6-only cell lines (see Table S9 in the supplemental material). In general, coexpression of E7 does not result in the addition of interactors with high NWD-scores to the list of E6 binding proteins or the loss of interactors from the list. Some NWD-scores (e.g., the NWD-score corresponding to CBP for HPV16 E6 or HERC2 for HPV33 E6) increase with the addition of E7, but the majority of the proteins detected in complex with E6 in the presence of E7 are the same as those detected in the absence of E7. We do note that there are approximately twice as many E6 peptides detected by the mass spectrometer when E7 is present versus when it is absent. Thus, the primary effect of E7 coexpression on E6 may be to increase the steady-state level of E6 in cells. This would in turn explain the increase in spectral counts and NWD-scores for the E6 interactors that we observed in the E6-plus-E7 cells.

The Ccr4-Not complex binds to HPV E6 proteins from genus beta, species 2. Other patterns in the comprehensive data set indicated that some genus beta E6 proteins participate in PV species-specific interactions with cellular proteins. One striking example is the interaction of E6s from genus beta, species 2 (HPV17a and HPV38 in this study), with 10 subunits of the Ccr4-Not complex (Fig. 2A, middle panel). Ccr4-Not is a multiprotein complex conserved from yeast to humans that is present in 1- and 2-MDa forms (reviewed in reference 13). The 10 subunits we detected (CNOT1, CNOT2, CNOT3, CNOT4, CNOT6L, CNOT7, CNOT9/RQCD1, CNOT10, C2orf29, and TNKS1BP1) are those that form the smaller 1-MDa core Ccr4-Not complex. Additional associated proteins are thought to contribute to the larger 2-MDa form. Ccr4-Not functions as one of the major cellular mRNA deadenylases and is associated with other enzymatic activities, including a ubiquitin ligase function. We confirmed the interaction of HPV17a and HPV38 E6s with CNOT1, CNOT2, and CNOT3 by immunoprecipitation-Western blot analysis (Fig. 5A). As in the immunoprecipitation-MS/MS analysis, interaction of Ccr4-Not subunits was more readily detected for HPV17a E6 than for HPV38 E6.

HPV92 E6 binds to a unique subset of cellular proteins. HPV 92 is the sole member of genus beta, species 4, identified to date. In keeping with its unique position in the HPV phylogeny, we observed that its E6 protein binds to a unique subset of cellular proteins (Fig. 2A, bottom panel). Several of these interactors also bind to one another, including both subunits of the HIF1 heterodimer: HIF1α and ARNT (HIF1β) (67). We identified four components of the centrosome, AZI1 (CEP131), CEP152, CEP63, and KIAA1712 (CEP44), as well as the microtubule binding protein KIAA1543 (CAMSAP3) in complex with HPV92 E6. Several proteins involved in cell adhesion, including JUB, AAMP, and the bovine papillomavirus type 1 (BPV1) E6-interacting protein Paxillin, bound to HPV92 E6, as did SLC12A8, a cation transporter that regulates keratinocyte proliferation, and DUSP3, a negative regulator of mitogen-activated protein kinase (MAPK). DUSP3 and HIF1 are of note because MAPK6 and HIF1AN have been recently shown to bind to E6AP for as-yet-unknown functions (42), suggesting that HPV92 E6 perhaps targets some of the same pathways as a high-risk HPV E6 but through other cellular protein interactions. We validated the interaction of Paxillin and of HIF1α with HPV92 E6 by immunoprecipitation-Western blot analysis (Fig. 5B) but note that the remaining interactions have yet to be validated.

HPV16 E6 mutant proteins exhibit distinct binding patterns. Several variants of HPV16 E6 have been constructed and characterized in a range of studies from different laboratories. To generate a comprehensive characterization of some of the most frequently used HPV16 E6 mutants, we chose three mutants of the HPV16 E6 protein, including one that is deficient in binding to p53 (8S9A10T), one that exhibits reduced binding to E6AP

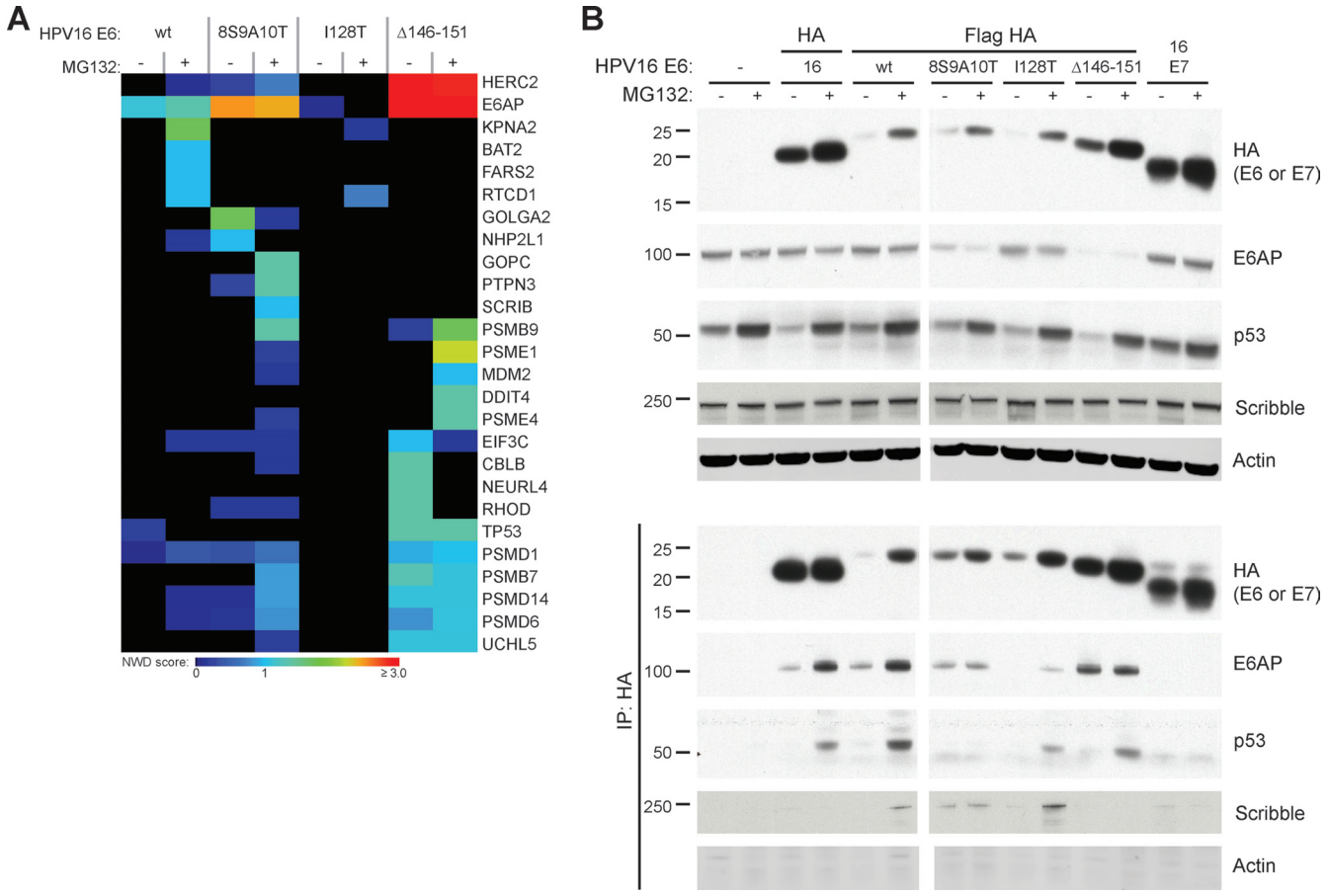

FIG 6 HPV16 E6 mutant proteins exhibit distinct binding profiles. (A) Heat map representing protein-protein interactions identified by immunoprecipitation-MS/MS and CompPASS analysis when the wild type (wt) or one of several mutant forms of FlagHA-HPV16 E6 was used as a bait. Colors in the heat map represent NWD-scores, where an NWD-score ≥ 1 defines a high-confidence interaction. Cells were treated for 4 h with 30 μM MG132 (+) or DMSO control (−) prior to harvest. HCIPs were arranged using a Manhattan distance hierarchical clustering analysis. (B) N/Tert-1 cells expressing the wild type or mutant HA-HPV16 E6, FlagHA-HPV16 E6, or HPV16 E7-FlagHA were treated for 4 h with 30 μM MG132 (+) or DMSO control (−), harvested, and subjected to immunoprecipitation with HA antibody. Immunoprecipitates were separated by SDS-PAGE and Western blotted using antibodies to HA, E6AP, p53, Scribble, or actin. Top panels, whole-cell lysates. Bottom panels, anti-HA immunoprecipitate.

(I128T), and one deleted for the 6-aa C-terminal PDZ binding domain (Δ146–151) (32, 33, 40).

To better understand how the profile of PPIs exhibited by wild-type HPV16 E6 changes when these mutations are introduced, we conducted an unbiased mass spectrometry analysis for the HPV E6s from different types in the same way as described above. Due to the availability of viral vectors when the 16E6 mutant retroviruses were constructed, the E6s used here (wild type and mutant) were tagged at their N terminus with both Flag and HA epitope tags. The wild type and each of the three mutant HPV16 E6s were stably introduced into N/Tert-1 cells and processed as previously described for immunoprecipitation-MS/MS and CompPASS analysis (Fig. 6A). This identified 26 HCIPs that interact with one or more of the HPV16 E6 variants tested (see Tables S6–S8 in the supplemental material).

These data and the subsequent validation of several PPIs by immunoprecipitation-Western blot analysis (Fig. 6B) confirmed several of the previously reported behaviors of these mutants. When cells were treated with MG132, each form of the protein tested bound to p53 except for the 8S9A10T mutant. HPV16 E6 I28T bound to E6AP only very weakly, with a minimal amount of binding detectable only in the presence of MG132. In the immunoprecipitation-MS/MS analysis of HPV16 E6 I128T, the loss of binding to E6AP resulted in a corresponding loss of binding to its binding partner HERC2. PDZ domain proteins, including PTPN3, Scribble, and GOPC, were detected in complex with one or more of the forms of HPV16E6 but were not detected with NWD-scores ≥ 1 for the PDZ binding domain mutant (Fig. 6A). Loss of binding to Scribble by the Δ146–151 mutant was confirmed in the immunoprecipitation-Western blot experiment (Fig. 6B). Thus, we have validated several behaviors of these HPV16 E6 mutants that have been reported in the literature, and we provide these data as a resource for others who use these forms of HPV16 E6.

E6AP mediates binding of E6 to the proteasome. Two subunits of the proteasome were detected in the initial analysis of 16 different E6 proteins (Fig. 2A), and seven proteasome subunits were recovered with at least one of the HPV16 E6 variants (Fig. 6A). Together with multiple studies indicating that E6AP binds to the proteasome (7, 34, 42, 53, 59, 68), this suggested that E6AP might be the molecular link connecting E6 to the proteasome. We reanalyzed the two data sets presented so far with slightly relaxed

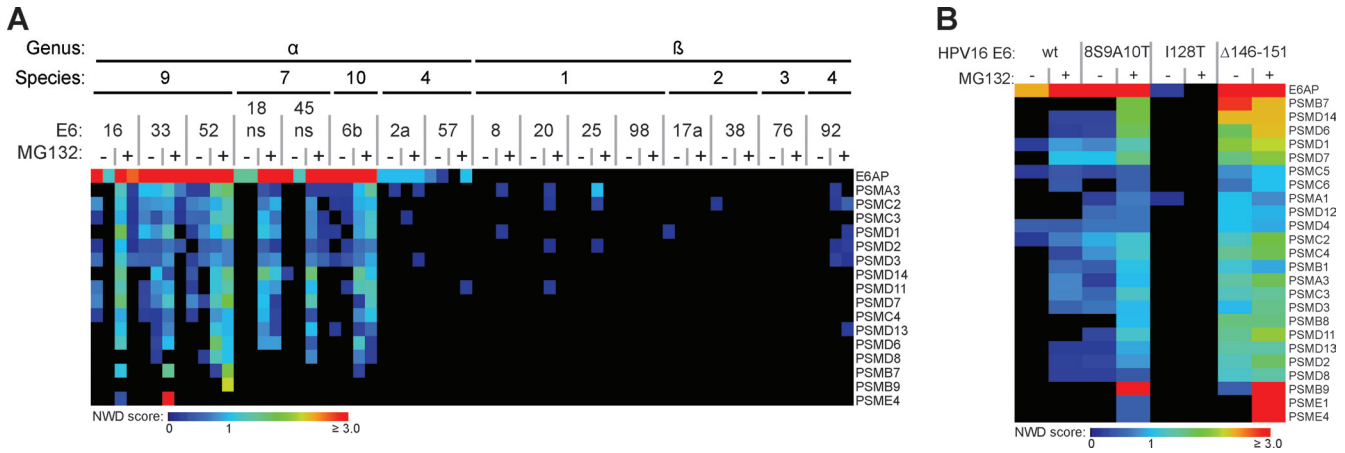

FIG 7 E6AP mediates the interaction of E6 with the proteasome. (A) Heat map representing protein-protein interactions identified by immunoprecipitation-MS/MS and CompPASS analysis when one of 16 unique HPV E6 proteins was used as bait. Colors in the heat map represent NWD-scores, where an NWD-score ≥ 1 defines a high-confidence interaction. NWD-scores are the normalized version of the CompPASS WD-score, which in this analysis was considered significant when it was ≥95% of scores representing the interactions in the CompPASS stats table. Each column in the heat map represents a replicate experiment. Cells were treated for 4 h with 30 μM MG132 (+) or DMSO control (−) prior to harvest. E6 baits were ordered across the top of the map according to the established HPV phylogeny (6); HCIPs were arranged using a Manhattan distance hierarchical clustering analysis. Only E6AP and subunits of the proteasome are shown as interactors. (B) Heat map representing protein-protein interactions identified by immunoprecipitation-MS/MS and CompPASS analysis when the wild type or one of several mutant forms of FlagHA-HPV16 E6 was used as bait. Colors in the heat map represent NWD-scores, where an NWD-score ≥ 1 defines a high-confidence interaction. NWD-scores are the normalized version of the CompPASS WD-score, which in this analysis was considered significant when it was ≥95% of scores representing the interactions in the CompPASS stats table. Cells were treated for 4 h with 30 μM MG132 (+) or DMSO control (−) prior to harvest. HCIPs were arranged using a Manhattan distance hierarchical clustering analysis. Only E6AP and subunits of the proteasome are shown as interactors.

statistical limits (accepting a protein as an HCIP if its CompPASS scores were ≥95% of the scores present in the stats table rather than applying the 98% limit used in the initial analyses) and selected the proteasome subunits that now scored as HCIPs (Fig. 7). Under these conditions, 16 unique subunits of the proteasome were identified in complex with at least one of the E6s tested. The interactions with NWD-scores ≥ 1 were nearly exclusive to the high- and low-risk E6s that bound E6AP with high NWD-scores. Consistent with this idea, the reanalysis of the HPV16 E6 mutant data revealed interactions with 24 different proteasome subunits, but these were lost with the introduction of the I128T mutation that limits E6 binding to E6AP (Fig. 7B). Together, these data support the idea that E6 binds to the proteasome via its interaction with E6AP.

A subset of beta-HPV E6 proteins stabilize p53. Several of the observations described above prompted us to conduct a more-detailed analysis of the properties of the beta-HPV E6 proteins included in our study. First, we examined the steady-state level of p53 present in the N/Tert-E6 stable cells (Fig. 8A). HPV16 E6 cells were included as a control and, as expected, expressed less p53 than the parental cells without E6. Steady-state levels of p53 were elevated in cells expressing HPV17a E6, HPV38 E6, and HPV92 E6. To determine whether this increased level of p53 was post-translational, we examined p53 stability in the presence of various

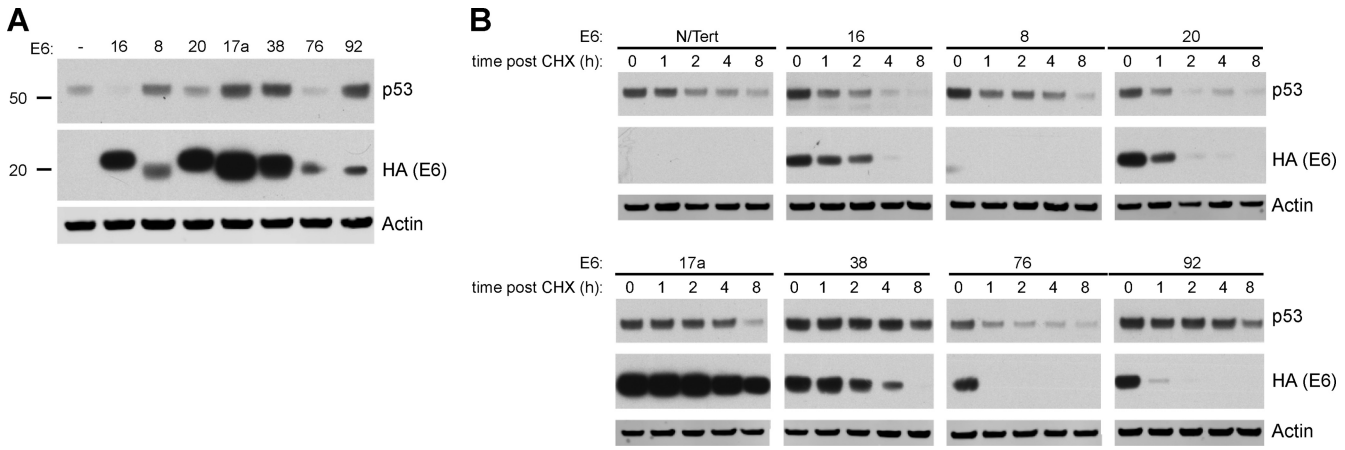

FIG 8 p53 is stabilized in cells expressing a subset of the beta HPV E6 proteins. (A) Western blot of p53 expression in N/Tert-HA-E6 stable cell lines. (B) Control cells or N/Tert-1 cells stably expressing HA-HPV16 E6 were treated with 40 μg/ml cycloheximide (CHX) and harvested at the indicated time points. Lysates were analyzed by Western blotting using antibodies to p53, HA, and actin.

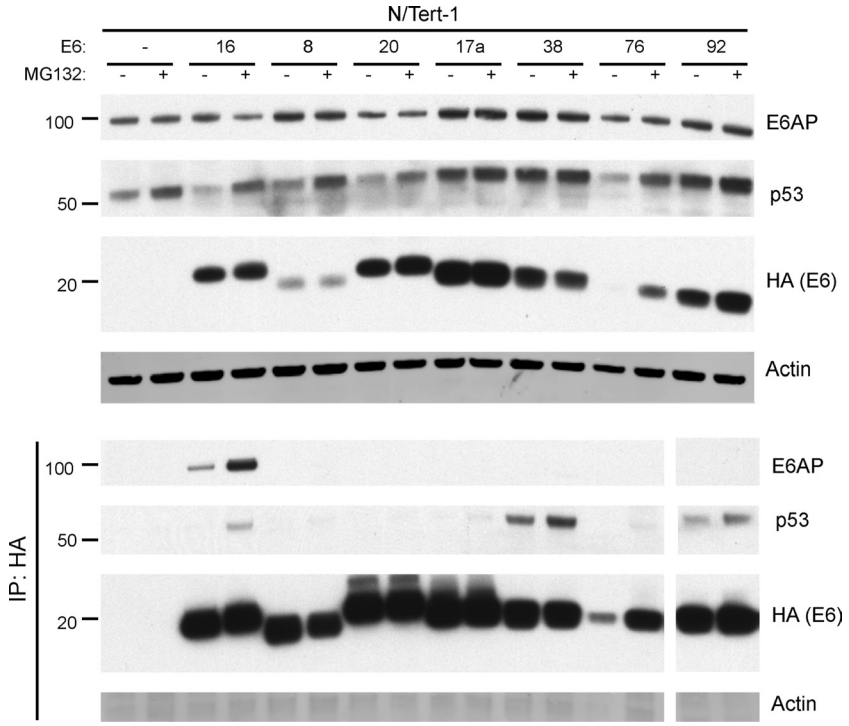

FIG 9 p53 is bound by HPV38 and HPV92 E6. N/Tert-1 cells expressing HA-E6 were treated for 4 h with 30 μM MG132 (+) or DMSO control (−) and subjected to immunoprecipitation with HA antibody. Immunoprecipitates were separated by SDS-PAGE and Western blotted using antibodies to HA, E6AP, p53, and actin. Top panels, whole-cell lysates. Bottom panels, anti-HA immunoprecipitate.

E6 proteins using Western blot analysis of protein levels after cycloheximide treatment of the N/Tert-E6 stable cells (Fig. 8B). Although (as shown in Fig. 8A) the levels of p53 at $t = 0$ did differ from one another rather significantly, here we present exposures of the p53 Western blots chosen such that the starting levels of p53 among the cell lines appeared comparable. The level of p53 in N/Tert-1 cells not expressing E6 dropped measurably over the 8-h time course in a manner consistent with the published ~4-h half-life of p53 in human keratinocytes (25). As expected, the presence of HPV16 E6 shortened this half-life, resulting in a more rapid degradation of p53 consistent with the reported 15- to 30-min half-life of p53 in the presence of HPV16 E6 (25). There was a marked stabilization of p53 present in three of the E6-expressing cell lines (N/Tert HPV17a, -38, and -92 E6). Thus, the elevated levels of p53 initially observed in these three cell lines were due to a posttranslational stabilization. This has been reported previously for cells that express both HPV38 E6 and E7 (1), but the expression of HPV38 E6 alone has not been shown to be sufficient for the stabilization, and neither has it been demonstrated for other beta HPV E6 proteins. We do note that, in some of the cases in which p53 was stabilized in the beta E6 (e.g., HPV92 E6)-expressing cell line, the E6 was turned over much more rapidly than the stabilized p53. We cannot rule out that additional or indirect effects contribute to the stabilization of p53 in these cells, and we are continuing to investigate this observation.

HPV38 E6 and HPV92 E6 bind to p53. Since the initial immunoprecipitation-MS/MS data also suggested that at least two genus beta HPV E6 proteins (HPV38 and HPV92 E6) bound to p53 (Fig. 2), and since we had begun to observe effects of the beta type E6s on p53 stability, we examined the binding of p53 to six different genus beta E6s (Fig. 9). In this experiment, HPV16 E6 in complex with p53 was detectable only when the proteasome was inhibited by MG132 treatment; HPV16 E6 also recovered E6AP in the presence and absence of MG132. Consistent with the immunoprecipitation-MS/MS data, both HPV38E6 and HPV92 E6 bound to p53, but neither one bound to E6AP. Both beta E6s that bound to p53 did so in both the presence and absence of MG132.

DISCUSSION

Here we report a systematic, unbiased analysis of the cellular proteins that interact with the E6 proteins from 16 different HPV types. This analysis defines new specificities for previously known binding partners of E6s and identifies previously unreported interactions. The list of 153 candidate and validated cellular interactors includes proteins that bind to HPV E6s of a single genus and to E6s from at least one distinct HPV species or type.

One striking example of a genus-specific interaction is the binding of genus alpha HPV E6s to E6AP and genus beta HPV E6s to MAML1. As both MAML1 and E6AP interact with E6 protein via their LXXLL motif (10, 11, 60), we propose that subtle genus-specific differences in the LXXLL binding sequence in E6 proteins result in this distinction (48). It is interesting to speculate whether the evolution of such a difference may have been one of the early events allowing a distinction between the mucosal and cutaneous tropisms of the two genera. E6 binding to MAML1 is potentially a key determinant of the ability of beta HPVs to replicate in skin, since Notch signaling in keratinocytes promotes differentiation and withdrawal from the cell cycle (41, 51). We note that E6AP has been previously reported to bind BPV1 E6 (8, 11, 66), but we speculate that this interaction was likely due to overexpression of

one binding partner or to *in vitro* artifacts and does not reflect physiological binding in a normal cellular environment.

A less general interaction is that of E6s with the acetyltransferases CBP and p300. These factors bound to HPV16 E6 (genus alpha, species 9) and to HPV8, -20, and -25 E6s (genus beta, species 1) but not to other alpha-9 virus types or to the other beta-1 type in the study. This is perhaps consistent with the reports discussed earlier that suggest that the function of the 16E6-CBP/p300 interaction is different from that of the beta species 1 E6-CBP/p300 interaction (23, 49, 61, 71) and suggests that the ability of E6 to bind these enzymes arose separately during HPV evolution.

In addition to these observations regarding binding specificities, our analysis has also identified previously unreported interactions. One of these is the interaction of E6s from genus beta, species 2, with 10 core components of the Ccr4-Not complex. To our knowledge, this is the first report of a virus-encoded protein binding to Ccr4-Not. The complex is primarily understood as a deadenylase conserved from yeast to humans, and as such it impacts several aspects of mRNA metabolism. In addition, it has an ubiquitin ligase function that has provided functional links to ubiquitylation and the proteasome (3). These and additional, less-well-characterized functions mean that the activities of Ccr4-Not in the cell are complex, and a series of experiments would be required to determine the downstream effects of the E6 interaction with Ccr4-Not.

Some of the highly specific interactions between HPV92 E6 (genus beta, species 4) and cellular proteins provide more functional clues. HPV E7 proteins from genus alpha have been shown to influence centrosome-related events, and it is possible that similar activities are provided by E6 in HPV92. The interaction of E6 with Paxillin is best understood for BPV1, in which case it disrupts actin cytoskeleton and focal adhesion components and may contribute to the transformation function (62, 63). Disruption of focal adhesion proteins by viruses is fairly common, and it is possible that this interaction reveals this function in HPV92 E6 as well. Neither HIF1α nor ARNT (HIF1β) contains an LXXLL motif, and it is possible that the interaction with HPV92 is mediated by another factor. Further studies to understand the downstream effects of these interactions and of the binding to HIF1 are required.

Finally, this study identified proteins that were previously identified only in complex with genus alpha HPV E6s but that we showed bind to some of the genus beta HPV E6s. One example is the binding of proteins containing PDZ domains to genus beta E6s. We note several examples of these interactions and validate the interaction of HPV38 E6 with PTPN13 (FAP1). PTPN13 is a protein tyrosine phosphatase that has not yet been clearly characterized as an oncogene or a tumor suppressor. It can inhibit tyrosine kinase signaling, suggesting growth inhibition, but can also inhibit Fas-mediated apoptosis (20). HPV16 E6 has been shown to induce the loss of PTPN13, and this interaction has been proposed to promote anchorage-independent growth of HPV16-positive cells (22, 58). In these reports, HPV16 E6 bound to PTPN13 after GST-tagged HPV16 E6 was incubated with 293 cell lysates *in vitro*, but in our cell-based system, we detected only the HPV38 E6-PTPN13 interaction.

The high-risk HPV C-terminal PDZ binding sequence is the conserved type I PDZ binding motif (PBM) X-T-X- ϕ_{COOH}, where ϕ represents a hydrophobic amino acid (in this case, L or V). No similar sequence is present at the C termini of the beta HPV E6s (Fig. 1A), but the range of sequences that bind to PDZ proteins is in fact much broader than had previously been appreciated (64), and there are four or more rather degenerate C-terminal PDZ binding sequences as well as internal PDZ binding sequences (39). It is also possible that the interaction between beta E6s and these PDZ domain proteins is not mediated by a classical C-terminal interaction but rather by binding via a protein intermediate or by binding to another sequence in the PDZ protein. Both the function and the nature of interactions between beta E6s and PDZ domain proteins have yet to be determined. It should be noted that interactions with proteins containing PDZ domains are not restricted to oncogenic viruses, that these interactions may contribute important functions to the replication of viruses even in the absence of a tumorigenic function (29), and also that HPV8 E6 was recently shown to inhibit expression of the PDZ protein Syntenin 2 at the level of transcription (36).

Binding to p53 is the other beta-HPV E6 interaction reported here that was previously thought to be restricted to the high-risk alpha HPV E6s. We demonstrate that at least two genus beta E6s, from HPV38 and HPV92, bind to p53 both with and without inhibition of the proteasome. Cells expressing either of these E6s, or HPV17a E6, exhibit elevated levels of p53, and we show that this increase in p53 is posttranslational, leading to an increase in the half-life of the protein. This is consistent with the reported studies by the Tommasino laboratory that found p53 stabilization in cells that express both HPV38 E6 and HPV38 E7 (1). As with the observations introduced earlier, the effects of p53-beta HPVE6 binding need to be investigated further.

Taken together, the data presented here represent a comprehensive analysis of the binding of cellular proteins to the HPV E6 proteins encoded by 16 diverse virus types. This is the first report describing many of these interactions, and, in addition, it expands our understanding of previously reported E6 binding partners. Most importantly, these data are a resource in the HPV field that will allow a wealth of functional studies with the potential to explain multiple aspects of HPV biology.

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